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(54) Identification of the origin of fruit or fruit juice

(57) The method is for the identification of the source genus, species, or variety of fruit tissue or juices derived therefrom and comprises (a) amplifying nucleic acids present in the tissue or juice using PCR, (b) comparing DNA obtained therefrom with that obtained from fruit tissue or juice of known genus, species or variety and (c) relating the presence of elements of a known genus, species or variety DNA to the presence of tissue or juice derived from that genus, species or variety. Preferably the random amplification of polymorphic DNA (RAPD) is applied to DNA from the tissue or juice. The nucleic acids are analysed using hybridisation probes or DNA profiles. Oranges, apples, lemons, limes, grapefruit, banana, kiwi fruit, passion fruit, paw-paw, peach, pineapple and plum may characterised.

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IDENTIFICATION OF FRUIT JUICE COMPONENTS.

The present invention relates to a method for identifying the origin of fruit derived tissues and extracts; particularly fruit juices, which may be in the form of pure or diluted liquid juices, pulps, purees, wines, jams or yoghurts, or other fruit juice products intended for human consumption; to nucleic acid primers and probes for use in this method and to test kits containing them.

Adulteration of fruit juices is a major problem, with a number of adulterants being used by unscrupulous producers to increase profits. Such adulteration renders it necessary to carry out authenticity testing on these juices and on fruit pulps and purees used in the production of food products such as wines, jams and yoghurts. A still further need for testing arises in the area of nursery stock procurement when verifying the genetic make-up of trees and bushes.

Particular problems vary from state to state; for example in the United States the products of the citrus hybrid 'Ambersweet' are sold as orange products, but in Europe Trading Standards Statutes do not permit this. Major retailers of juice related products thus have a requirement for carrying out athenticity testing, either by themselves or by their suppliers. Furthermore different fruit products attract different import duties, making source identification necessary by the customs authorities.

Analytical chemists have developed a range of testing techniques and some of these have a highly sophisticated nature. For example, it is known to characterise a variety of tissues using 'genetic finger -printing' whereby nucleic acids are isolated from the material to be typed, and then subjected to detection and identification. Conventionally one of two techniques is used depending on whether 'marker' or 'conserved' sequences are present in the test material. Particularly used are nucleic acid probe based methods and PCR

polymerase chain reaction (PCR) based methods. A range of commercially available nucleic acid probes which bind to conserved nucleic acid sequences have been developed for the detection of bacterial pathogens. Examples of these include colorimetric (Genetrak Systems) and chemiluminescent Gene probe (Eugenetics UK) systems for Salmonella and Listeria monocytogenes. Use of such probing to type and identify humans is well documented with gene probe techniques.

Amplification of the target conserved sequences by PCR prior to detection of the amplified PCR product is another recently developed powerful technology that is proving invaluable in clinical and forensic science. The scope of the PCR technique is considerable in other industries, including the food production, agriculture and water industries. The technique is capable of generating microgram quantities of the target sequences starting from nanogram levels within a matter of 3 to 4 hours. In the case of microbial pathogen analysis considerable time-saving is achieved by using PCR as opposed to classical growth based methods which can take days to achieve the same result.

The present inventors have now determined that nucleic acids may be isolated from a range of fruit, and particularly from fruit juices, these being those fluids derived from whole fruit and being sold with or without tissue content. Furthermore, they have determined that such DNA can be probed or subjected to PCR such that characterisation of the fruit source may be carried out, and have further determined specific probe and primer sequences suitable for such characterisation. The application of these techniques to fruit juice allows rapid detection of adulteration without the need to have a sample of the fruit tissue it is derived from.

Thus the present invention provides a method for the identification of the source genus, species, or type of fruit tissue or juices derived therefrom comprising (a) analysing nucleic acids present in the tissue

or juice (b) comparing DNA profiles obtained therefrom with those of profiles obtained from fruit tissue or juice of known genus, species or variety and (c) relating the presence of elements of a known genus, species or variety DNA profile to the presence of tissue or juice derived from that genus, species or variety.

This invention is based upon the inventors determination that while levels of nucleic acid present in many juices are insufficient to allow application of direct DNA probing, there is sufficient to allow application of PCR based techniques to produce sufficient DNA. Furthermore they have found that this nucleic acid is such that it is characteristic of the fruit, and that the rootstock DNA does not appear in scion samples in quantities that can be detected. The inventors have further determined the sequence of several PCR primers that provide distinct profiles when applied to fruit tissue or juice nucleic acid.

Most advantageously the DNA profile is obtained by application of PCR to amplify DNA sequences present in the fruit DNA whereby characteristic DNA products are obtained that can be compared with known products for identification purposes. For example, the present inventors have found that comparison of DNA profiles from orange and grapefruit, fruits of the same genus, shows distinct differences to be present, and that mixtures of these show a combinatorial pattern.

Particularly it is found that application of random amplification of polymorphic DNA (RAPD) to extracted DNA provides for the successful characterisation of fruit tissues and juices with sufficient differentiation to allow identification of the origin of components of blends. Use of the preferred technique allows ready distinction between different species of fruit crops and, at lower frequency, different varieties of fruit, such as orange, grapefruit and apple varieties. The fact that the inventors have determined that rootstock does not affect the DNA profile of the scion, and thus that genes do

not move between cells such as to demonstrate the reliability of the technique as applied to such 'bi-genomic' plants.

In a preferred method of the invention there is incorporated a procedure for extracting high quality DNA from intact fruit or processed fruit juice. For tissue this procedure involves freezing to provide a solid which is then mechanically transformed into a powder which then extracted with a solvent capable of taking up DNA. The freezing step conveniently comprises freezing the tissue in liquid nitrogen and the mechanical powdering conveniently comprises a grinding step. Extraction solvent is conveniently that of the cetyltrimethyammonium bromide (CTAB) and/or phenol chloroform methods conventional in the art. Using such techniques it is possible to obtain DNA suitable for profiling purposes from a wide variety of fruit including Navel, Valencia and satsuma oranges, red and Granny Smith apples, lemons, limes, grapefruit, banana, kiwi fruit, passion fruit, paw-paw, peach, pineapple, grape and plum,

Use of such extraction techniques provides variable DNA yield between different genus and species of fruit (see Table 1), but all are found to be sufficient for the preferred RAPD.

TABLE 1

FRUIT	COUNTRY OF ORIGIN	DNA YIELD µg/g FW
Apple:		
Red	France	0.14
Granny Smith	France	0.98
Lemon	South Africa	1.71
Lemon	Argentina	0.54
Lime	USA	3.92
Grapefruit	South Africa	1.09
Banana	West Indies	0.27

Table 1 Contd.

Kiwi fruit	New Zealand	5.58
Passion fruit	Kenya	9.48
Paw-paw	Brazil	4.20
Peach	Italy	2.71
Pineapple	Costa Rica	49.92
Plum	England	1.46
Mango	Kenya	5.50
Galia melon	Israel	7.82
Orange:		
Navel N1-625	South Africa	3.70
N5-J1207	Morocco	3.64
Juice	Morocco	0.128/ml
Conc.Juice	Morocco	6.16
Proc.Juice	Morocco	0.094/ml
N6-1207.1	Spain	3.83
N6-1207.2	Spain	1.25

Particular primer sequences that have been found to produce satisfactory profiles for the identification of fruit genus, species or varieties are also provided and are those which are targeted to initiate PCR amplification at the 5' terminal of sequences given as SEQ ID No 1 to 6 described below:

SEQ ID No 1: GGTCTAGAGG
SEQ ID No 2: ACTCCAGTCC
SEQ ID No 3: TGGAGGGCTC
SEQ ID No 4: AGACGGCCCT
SEQ ID No 5: GTCCCAGCAG
SEQ ID No 6: TGTCGGTCGT

Thus primers targeted at these sequences may vary from complementary sequences to the target sequences at the 5' end of the target, but should allow hybridization at the 3' end. Thus typical primer sequences comprise the sequences SEQ ID No 7 to 12 below and preferably consist of these.

SEQ ID No 7: CCTCTAGACC
SEQ ID No 8: GGACTGGAGT
SEQ ID No 9: GAGCCCTCCA
SEQ ID No 10: AGGGCCGTCT
SEQ ID No 11: CTGCTGGGAC
SEQ ID No 12: ACGACCGACA

Using these primers it is possible to characterise and therefore identify a great number of fruit tissues, and more significantly fruit juices, as set out in the Table 2 below:

The preferred technique used to produce RAPD profiles is that which is conventional in the art of PCR analytical techniques, eg. that as described by Mazurier et al (1990), Nucleic Acid Res., 18, 6531-6535 and Mazurier and Werners (1992), Res. Microbiol, 14, 260-262.

In a further aspect of the present invention there are provided test kits comprising oligonucleotides comprising sequences complementary to SEQ ID No 1 to 6, particularly comprising respective sequences of SEQ ID No 7 to 12, particularly consisting of the latter sequences.

In a still further embodiment of the invention there are provided test kits comprising the oligonucleotides as described above further comprising comparative patterns of known fruit derived from one or more RAPD using respectively each of these primers, together or alone.

The oligonucleotides described above are capable of being used as PCR primers for RAPD analysis of fruit tissue and fruit juice are further

capable of being used to provide oligonucleotide hybridization probes which are themselves useful in analysis and characterisation of fruit tissue and fruit juice nucleic acid by Southern blotting hybridization.

When used as PCR primers the oligonucleotides may be increased in length to allow increased stringency hybridization conditions to be used and thus to increase the specificity of the method. For example, the SEQ ID No 12 primer may be extended to provide forward and reverse primers capable of binding to target DNA under more stringent conditions by addition of one or more bases to its 3' end as set out in the following sequences;

Forward primer sequence: (Bases 1-10 are those of SEQ ID No 12; the primer is enlarged by adding a desired number of bases in the order of bases 11-51 of the sequence below).

5'-ACGACCGACA AACGATGAAA ATACTCACGG CTGTGCATAA AGACGCGCAA TATTAGAGAA GGTGCGTTGA TTTGGATTTG GCGT-3'

Reverse primer sequence: (Bases 1-10 are those of SEQ ID No 12; the primer is enlarged by adding a desired number of bases in the order of bases 11-51 of the sequence below).

5'-ACGACCGACA CCAAAACCAA AGTCAAGAAC TTCGGCGCAA TAGAGCATAC TTGCAATGAA TTTCACATAT GATCACTA-3'

It will be clear to those skilled in the art that primers consisting of any 5 or more bases of these sequences, preferably any 10 or more, most preferably any 15 or more, may be used to amplify orange DNA to produce an approximately 1kb fragment that is characteristic of orange species. The specificity of such primers will be increased by increasing their length corresponding to the aforesaid sequences. SEQ ID No 12 is known (Operon Technologies), but oligonucleotides derived from the rest of the sequence are new.

Thus these products using such further derived primers may be used

themselves as Southern blot hybridization probes. Particularly these probes comprise individual labelled PCR products of the PCR reaction of fruit derived tissues using PCR primers of sequence as described above.

One preferred set of polynucleotide/oligonucleotide probes are those derived by carrying out a PCR reaction on DNA derived from Florida 'Ambersweet' orange/grapefruit hybrid tissue, particularly the leaves, using the only the primer SEQ ID No 12 to initiate the amplification. The polynucleotide, of approx 1Kbp in length, is unique to Ambersweet.

A further preferred polynucleotide for the purpose of labelling and using as a probe is an Ava1/Bg1II fragment obtainable from this 1Kb polynucleotide and is approximately 200 bp in length. The preferred 200 bp product hybridizes specifically with orange and Ambersweet derived genomic nucleic acid but not to that from grapefruit or lime.

A second group of such polynucleotides provided by the present invention is that made available by use of SEQ ID No 11 to amplify DNA derived from any of the orange cultivars referred to herein. The product here is of about 1kb and a 500bp fragment can be derived from this using EcoRV/Bg1II. Again neither the product or the 500bp fragment has been found in DNA derived from grapefruit or lime. Further polynucleotides can be derived by cleaving this fragment that similarly are specific to orange DNA.

A further aspect of the present invention provides a method for production of the 200 bp polynucleotide of the invention comprising cloning the approximately 1 Kb PCR derived product obtainable using SEQ ID No 12 to amplify 'Ambersweet' DNA, particularly 'Ambersweet' leaf DNA, into a vector system, and cleaving the required approximately 200 bp product from that using Ava1 and Bg1 II, followed by a labelling step. Labelling will be by any of the conventional biological, chemical or radioactive label incorporating methods known

in the art.

The method, oligonucleotides and kits of the invention will now be described by way of illustration only, by reference to the following Examples. Further embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

EXAMPLES

EXAMPLE 1: Procedure for isolation of DNA from commercially available fruit juice.

Initial attempts to isolate DNA from 100ml of commercial fruit juice were made but the optical density values (260nm) were negative and no bands of genomic DNA could be visualised following agarose gel electrophoresis. Isolation was then attempted using 400ml of fruit juice, wherein positive optical density values were obtained but genomic DNA could not be observed following gel electrophoresis. PCR products were obtained implying that although DNA could not be observed on the agarose gels sufficent was available for amplification. Such volume requirement will of course depend upon the origin and prior processing of the juice.

The pH of 400ml of commercial juice was adjusted to pH 7.0 using 8.33N sodium hydroxide, then centrifuged at 10,000g to produce a supernatant and a solid phase, and the two phases separated. DNA was isolated from the supernatant by adding 3M ammonium acetate and isopropanol to cause its precipitation. The precipitate DNA was pelletized by centrifugation at 10,000g and treated with a cationic detergent solution (CTAB:cetyltrimethylammonium bromide) at a concentration of 0.8% w/v to reduce polysaccharide contamination and thus avoid inhibition of enzymes involved on cloning procedures. Chloroform was added to the treated DNA, the mixture centrifuged and two phases resolved. The aqueous phase containing DNA was removed and DNA precipitated with isopropanol. DNA was pelleted by centrifugation and the pellet resuspended in TE (Tris-HCl, Na_2EDTA).

Phenol/chloroform treatment was used to denature any proteins which formed a layer at the interface between the organic and aqueous phases, and the step repeated until no protein was left at this interface. Sevag (chloroform: isoamyl alcohol) treatment was carried out by adding isoamyl alcohol to prevent foaming of the mixture upon subsequent vortexing thus aiding separation of the two phases. RNAse was added to remove RNA, extraction carried out with phenol/ chloroform and Sevag as before, DNA precipitated with isopropanol and 3M ammonium acetate, centrifugation carried out to pellet DNA and the resultant pellet washed with 70% ethanol. The final pellet was vacuumed dry and resuspended in sterile distilled water.

EXAMPLE 2: Extraction of DNA from fruit tissue. 30 g fruit tissue was frozen in liquid nitrogen and ground to a powder for DNA extraction. TES (Tris-HCl, Na_2EDTA and SDS) extraction buffer was added to the thawed material for cell lysis whereby reduced nuclease activity was provided. 1M PAH (phenanthroline hydrate) and 1M DTT (Dithiotreitol) reducing agents were added to inactivate proteins and phenol added to cause their denaturation. The mix was centrifuged and the aqueous phase resulting treated as for the aqueous phase 'supernatant' produced after initial NaOH treatment in Example 1.

EXAMPLE 3: RAPD analysis of fruit tissue and juice derived DNA. RAPD was carried out using six DNA primers consisting of SEQ ID No 7 to 12 respectively (Operon Technologies) in separate amplifications, the primers acting at both ends of the target DNA to be analysed. Amplifications were carried out in a total volume of 25µl containing:

2.5µl of 10 x buffer (Mg^+ free)
0.1µl of dNTP mixture (each at 100mM)
2.0µl of 25mM $MgCl_2$
5.0µl of a single one of the 10 base oligonuleotide primers (3ng/µl)
2.5µl of genomic template DNA (10ng/µl)
0.2µl of Taq DNA polymerase (1 unit)
12.7µl of sterile distilled water

With the exception of genomic template, primer and Taq DNA polymerase, all the above constitute the core buffer which is prepared in bulk volume and stored at -20°C. All amplifications were performed using a Perkin-Elmer Thermal Cycler with the following temperature programme.

1 cycle of	3 minutes	at 94°C
	1 minute	at 36°C
	2 minutes	at 72°C
44 cycles of	1 minute	at 94°C
	1 minute	at 36°C
	2 minutes	at 72°C
Final cycle	10 minutes	at 72°C
	999	18°C

Amplification products were analysed by gel electrophoresis using a 1.4% agarose gel run at 150V (constant voltage) for 2 hours and using ethidium bromide staining to visualise profiles obtained.

A series of fruit tissue and juice derived DNAs was used to carry out PCRs in the presence of each of the single oligonucleotide primers described above as having sequences consisting of SEQ ID No 7 to 12.

The primer/DNA combinations gave either no product (NP), similar product profiles (+) for all DNAs tested, or distinct product profiles (-). The results of these PCRs are given in Table 2 below. Where gaps are given, the particular primer has no been applied. Thus those combinations shown as (-) are suitable for use in identification of specific DNA and thus particular fruit tissue/juice in RAPD application.

PRIMER SEQ ID No FRUIT	7	8	9	10
Red Apple	NP	-		
Granny Smith	-	-		
South African Lemon	NP	+		
Argentinian Lemon	NP	-		
Lime	-	-		
Grapefruit	NP	+		
Banana	-	-		
Valencia orange V3-805	-	+		
Navel N5-1207			+	+
'' N5-PJ1207 Processed Juice			+	+
'' N5-J1207 Juice			+	-
'' N5-CJ1207 Concentrated Juice			+	+

In addition to these results the oligonucleotide primer having the sequence of SEQ ID No 10 was applied to PCRs of tissue of a number of varieties of Navel, Valencia and satsuma oranges. All but N2-625 gave the same profile out of the following Accessions. N1-625, N2-625, N3-805, N3-625, N4-805, N5-1207, N6-1207-1, N6-1207-2, V1-625, V2-805, V3-805, V4-1016, V5-805, V6-1016, V7-1016, VL-1207, S1-1207.

It was noted that all tissue or juice samples could be caused to give a profile by use of one or other of the primers provided. Some

primers gave similar profiles for related species; eg. SEQ ID No 8 and 9, Differences could be determined between different Accessions of Navel oranges, and between Granny Smith and red apples; eg. SEQ ID No 7.

The primers having sequences of SEQ ID No 11 and 12 were found to be particularly useful in distinguishing orange species from other fruit, or 'Ambersweet' orange and grapefruit hybrid from orange varieties.

EXAMPLE 4: Preparation of oligonucleotide probe capable of distinguishing oranges. A primer of DNA sequence SEQ ID No 12 was used to amplify a DNA fragment from 'Ambersweet' leaf derived genomic DNA using PCR; this product fragment being of approximately 1 kb long. The product was reamplified using 16 cycles and run on an agarose gel whereafter the polymorphic 1 kb band was cut out and purified using 'The Geneclean Kit' (Bio 101 Inc. USA). The quantity of DNA produced was then determined. The 1 kb fragment was then cloned into the pGEM-T plasmid vector system (Promega) and single and double restriction enzyme digests set up to relatively locate the enzyme sites present in the vector. The enzymes chosen, Ava1 and Bg1 II, do not affect sites native to the vector as sold. The approx. 200 kb Ava1/Bg1 II fragment oligonucleotide product was extracted from the rest of the insert, purified and labelled for use as a probe according to standard methods. Using Southern blot/hybridization analysis this probe was found to be capable of hybridizing to orange genomic DNA, but not that of grapefruit or lime.

EXAMPLE 5: Use of RAPD to distinguish grapes. The procedure of Example 3 was repeated on both black and white grape varieties using a number of primers, including those of SEQ ID No 9, 10 and 17. Use of a primer of SEQ ID No 9 gave identical RAPD patterns with both varieties, while a primer of SEQ ID No 12 gave different patterns for black and white and one of SEQ ID No 10 gave bands only with black grapes.

EXAMPLE 6: Use of RAPD to distinguish Sweetie interspecific hybrid from grapefruit. Primer of SEQ ID No 8 was used to produce RAPD DNA patterns from DNA samples from Sweetie, an interspecific hybrid, and from grapefruit. Several bands were produced with Sweetie DNA that did not develop with grapefruit DNA as template.

CLAIMS.

1. A method for the identification of the source genus, species, or variety of fruit tissue or juices derived therefrom comprising (a) amplifying DNA sequences in nucleic acids present in the tissue or juice using polymerase chain reaction, (b) comparing DNA obtained therefrom with that obtained from fruit tissue or juice of known genus, species or variety and, (c) relating the presence of elements of a known genus, species or variety DNA to the presence of tissue or juice derived from that genus, species or variety

2. A method as claimed in claim 1 wherein the nucleic acids are analysed using hybridization probing whereby the occurence of hybridization of fruit nucleic acids with a given probe is compared with that with known genus, species or variety nucleic acid, and such comparison used to determine the likely identity of the fruit.

3. A method as claimed in claim 1 or 2 wherein random amplification of polymorphic DNA (RAPD) is applied to DNA from the tissue or juice. and profiles provided used to compare the DNA with that of known profiles.

4. A method as claimed in any one of the preceding claims wherein prior to amplification and analysis the nucleic acid is extracted from fruit tissue by freezing the tissue to a solid, mechanically transforming the solid into a powder, and extracting the powder with a solvent capable of taking up DNA.

5. A method as claimed in claim 4 wherein the freezing step comprises freezing the tissue in liquid nitrogen and the mechanical transformation into powder comprises a grinding step.

6. A method as claimed in any one of claims 1 to 5 wherein prior to amplification and analysis the nucleic acid is extracted from fruit

juice by precipitation followed by cetyltrimethyammonium bromide (CTAB) and/or phenol chloroform treatment.

7. A method as claimed in any one of claims 3 to 6 wherein the RAPD is carried out using one or more primers targeted to initiate PCR amplification at the 5' terminal of one of the DNA sequences:

SEQ ID No 1: GGTCTAGAGG
SEQ ID No 2: ACTCCAGTCC
SEQ ID No 3: TGGAGGGCTC
SEQ ID No 4: AGACGGCCCT
SEQ ID No 5: GTCCCAGCAG
SEQ ID No 6: TGTCGGTCGT

8. A method as claimed in any one of claims 3 to 7 wherein the RAPD is carried out using one or more of primers comprising any one of sequences SEQ ID No 7 to 12:

SEQ ID No 7: CCTCTAGACC
SEQ ID No 8: GGACTGGAGT
SEQ ID No 9: GAGCCCTCCA
SEQ ID No 10: AGGGCCGTCT
SEQ ID No 11: CTGCTGGGAC
SEQ ID No 12: ACGACCGACA

9. A test kit suitable for use in identification of the genus, species or variety of a fruit tissue or fruit juice characterised in that it comprises an oligonucleotide including the sequence of any one of SEQ ID No 7 to 12.

10.A test kit as claimed in claim 9 further comprising comparative patterns of known fruit derived from one or more RAPD using respectively each of these oligonucleotides as primers, together or alone.

11.An oligonucleotide comprising five or more bases of the sequence

5'-AACGATGAAA ATACTCACGG CTGTGCATAA AGACGCGCAA TATTAGAGAA GGTGCGTTGA TTTGGATTTG GCGT-3'

12.An oligonucleotide comprising five or more bases of the sequence

5'-CCAAAACCAA AGTCAAGAAC TTCGGCGCAA TAGAGCATAC TTGCAATGAA TTTCACATAT GATCACTA-3'

13.An oligonucleotide as claimed in claim 11 or 12 characterised in that it comprises 15 or more bases of the given sequence.

14.A polynucleotide or oligonucleotide hybridization probe obtainable by performing a PCR amplification of fruit tissue or juice derived nucleic acid using one or more of the oligonucleotides of SEQ ID No 7 to 12 as primers and purifying or isolating the product.

15.A probe as claimed in claim 14 being obtainable from Florida 'Ambersweet' orange leaf derived genomic DNA, using only a primer of SEQ ID No 12 to initiate the amplification.

16.A probe as claimed in claim 15 obtainable by exposing the polynucleotide of claim 14 to Ava1/Bg1 II restriction endonucleases and isolating a fragment from the products of approximately 200 bp in length.

17.A probe as claimed in claim 16 obtainable from orange cultivar derived genomic DNA, using only a primer of SEQ ID No 11 to initiate the amplification.

18.A probe as claimed in claim 14 obtainable by exposing the polynucleotide of claim 17 to EcoRV and Bg1II restriction endonucleases and isolating a fragment from the products of approximately 500bp in length.

Patents Act 1977
Examiner's report to the Comptroller under Section 17
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Databases (see below)
(i) UK Patent Office collections of GB, EP, WO and US patent specifications.

(ii) ONLINE: WPI, CAS ONLINE, BIOTECH/DIALOG

Documents considered relevant following a search in respect of Claims :-
1-18

Categories of documents

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Category	Identity of document and relevant passages		Relevant to claim(s)
Y	EP 0534858 A1	(KEYGENE) see pages 2-3; Example 1	1-6
Y	EP 0337625 A2	(I.C.I.) see page 2, lines 43-49; page 3, lines 20-25, 51-58	1-6
Y	WO 92/11372 A1	(I.C.I.) see Example 2	1-6
Y	WO 90/05195 A1	(DNA PLANT TECHNOLOGY CORP) see Examples 6 & 7	1-6
Y	Letters in Applied Mocrobiology, Vol 14, 1992, pages 260-262 Mazwier et al. See Introduction		3
Y	Nucleic Acids Research, Vol 18. No 22, 1990 pages 6531-6535 Williams et al. See Discussion		3

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